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Metabolic synergies in the biotransformation of organic and metallic toxic compounds by a saprotrophic soil fungus

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Running head: Fungal biotransformation of hexachlorocyclohexane and vanadium

Abstract

The saprotrophic fungus *Penicillium griseofulvum* was chosen as model organism to study responses to a mixture of hexachlorocyclohexane (HCH) isomers (α-HCH, β-HCH, γ-HCH, δ-HCH) and of potentially toxic metals (vanadium, lead) in solid and liquid media. The *P. griseofulvum* FBL 500 strain was isolated from polluted soil containing high concentrations of HCH isomers and potentially toxic elements (Pb, V). Experiments were performed in order to analyse the tolerance/resistance of this fungus to xenobiotics, and to shed further light on fungal potential in inorganic and organic biotransformations. The aim was to examine the ecological and bioremedial potential of this fungus verifying the presence of mechanisms that allow it to transform HCH isomers and metals under different, extreme, test conditions. To our knowledge, this work is the first to provide evidence on the biotransformation of HCH mixtures, in combination with toxic metals, by a saprotrophic non-white-rot fungus and on the metabolic synergies involved.

Keywords: soil saprotrophic fungi, hexachlorocyclohexane, vanadium, biotransformation, metabolic phenotype, medium pH

Introduction

Hazardous persistent organic pollutants, e.g. pesticides, pharmaceuticals, explosives, and potentially toxic elements (PTEs), e.g. Pb, Cr, As, Sn, constantly enter ecosystems (waters, soils, and sediments) causing severe environmental and health problems (Polti et al. 2014; Wan et al. 2015). Anthropogenic processes associated with domestic, municipal, agricultural, industrial, and military activities represent the major sources while PTEs can also be released from natural geological processes such as weathering and volcanic eruption (Vargas-García et al. 2012; Griffith et al. 2015). Pollution due to human activities is often the result of extensive histories of multiple land use which creates sites containing mixed pollutants. More than 40% of the United States National Priority List sites are co-contaminated by organic (volatile and semi-volatile organic compounds) and inorganic pollutants (metals including radionuclides) while metals and mineral oil contribute jointly to around 60% of soil contamination and 53% of groundwater contamination in Europe (Sandrin and Maier 2003; Panagos et al. 2013). The co-occurrence of organic and metal pollutants is not only a threat to human and ecosystem health, but is also a challenge because the technologies required for remediation of polluted sites are different for each group of pollutants (Sandrin and Maier 2003; Zhu et al. 2012). As more than one third of contaminated sites are polluted by more than one type of contaminant, it is imperative to develop cost-effective and sustainable techniques that can transform organic compounds while also extracting PTEs or stabilizing them in non-toxic forms (Polti et al. 2014).

In recent years, several studies on microbial communities or on single microbes isolated from historically contaminated sites have shown their ability to tolerate, adapt and grow in the presence of organic compounds and PTEs (Alisi et al. 2009; Wasi et al. 2011). This suggests that bioremediation based on microbial activities is feasible for the recovery of such sites by transformation or immobilization of both organic compounds and PTEs (Zhu et al. 2012; Polti et al. 2014). Previous studies on microbial biotransformations of multiple contaminants have mainly concentrated on bacteria (Alisi et al. 2009; Wasi et al. 2011), but fungi can also represent ideal candidates for future challenges in complex multi-contaminated contexts. Fungi are ubiquitous chemoorganotrophic organisms, playing fundamental roles in ecological and geological processes (Gadd 2010; Gadd et al. 2012). As decomposers, pathogens, and symbionts (mycorrhizas, lichens), fungi provide fundamental ecological functions for ecosystems and human well-being (Mace et al. 2012; Lange et al. 2012). Fungi can transform a huge variety of organic substrates, including natural polymers such as cellulose, lignin, chitin and starch but also many anthropogenic products like pesticides, explosives and other xenobiotics (Gadd 2013; Harms et al. 2011). Due to their filamentous growth habit and ability to exude organic acids, protons and other metabolites, fungi are important biological weathering agents of rocks and mineral-based substrates (Gadd 2004; 2007). The potential of fungi to tolerate and transform both organic and inorganic pollutants has been highlighted in many studies which have also reported some unusual abilities shown by fungi isolated from contaminated soil (; Tigini et al. 2009; Ma et al. 2014; Mishra and Malik 2014). In fact, isolation of indigenous fungi could provide the best candidate organisms for bioremediation of polluted soil since they

already belong to an established soil microbial community, and are best adapted to the site conditions (Czaplicki et al. 2016).

In this research, we have examined tolerance to both organic and inorganic pollutants by a soil saprotrophic fungus, *Penicillium griseofulvum* Dierckx isolated from a historically polluted soil. The study area was the Italian National Site of Interest "Valle del Sacco" (Lazio, Italy) which is affected by high concentrations of multiple pollutants such as hexachlorocyclohexane, vanadium and lead (Ceci et al. 2012; Bernardini et al. 2016; Bernini et al. 2016). The aims of this research were to evaluate the tolerance/resistance of *P. griseofulvum* FBL 500 to different combinations of vanadium, lead, and isomers of HCH; to study the inorganic biotransformation in the presence of metals, and possible biomineralization phenomena; and to analyse biodegradation of an isomeric mixture of HCH, in the absence and in the presence of vanadium, and the impact of any synergistic effects on fungal metabolism.

Materials and methods

Organism, media, and growth conditions

A strain of *P. griseofulvum* Dierckx (FBL 500), obtained from the culture collection of the Fungal Biodiversity Laboratory (FBL) (Sapienza, University of Rome), was used in all the biotransformation tests. Czapek-Dox medium was used for all the experiments (; Ceci et al. 2015b, c). The strain is also preserved in the public mycological collection of Mycotheca Universitatis Taurinensis (MUT) as MUT 5854. The Czapek-Dox agar medium contained the following (g/l distilled water): $NaNO_3$, 3; K_2HPO_4 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01. All chemicals were purchased from Merck (Darmstadt, Germany).

The experimental design was composed of three different experiments, in which (A) tolerance/resistance responses and biotransformation of metals (V, Pb), (B) of HCH mixture of isomers, and (C) of the synergic effects of combination of V and HCH isomers were respectively investigated (see Table 1, Online Resource). The whole phenotypic response of this fungus to the different treatments was examined using the Phenotype MicroArray™ system (Pinzari et al. 2016). Sucrose and D-glucose (Difco, Sparks, MD, USA) were used as substrates for fungal growth (Table 1, Online Resource). The glucose concentration was 30 g/l in experiment A, in which *P. griseofulvum* FBL 500 was tested with metals (V and Pb compounds): this concentration was used following the same cultural conditions as in Ceci et al. (2015a, c) (Table 1, Online Resource). 5 g/l sucrose was used in experiment B to stimulate the biotransformation of HCH isomers as used in previous tests of biotransformation of β-HCH by *P. griseofulvum* FBL 500 (Ceci et al. 2015b) (Table 1, Online Resource). The same concentration was maintained in experiment C to study synergic interactions between the fungus and different combinations of V and HCH. In experiment A, prior to autoclaving, the medium pH was adjusted to 5.5 using concentrated HCl (Ceci et al. 2015a, c), while in experiments B and C, the pH was kept at 7 in order to prevent acidic variations of the medium pH, which could result in toxicity to *P. griseofulvum* FBL 500 during HCH biotransformation because of benzoate formation (Guillén-Jiménez et al. 2012; Ceci et al. 2015b) (Table 1, Online Resource).

In experiments A and C, prior to inoculation, 84 mm diameter discs of sterile cellophane membrane (Focus Packaging and Design Ltd, Louth, UK) sterilized by autoclaving in distilled water and were placed aseptically on the surface of the agar in each Petri dish (Ceci et al. 2015c). Growth of *P. griseofulvum* FBL 500 was evaluated by measuring diametric extension of the colony and by biomass yield since extension of the colony alone does not take into account the density of fungal mycelium (Ceci et al. 2015c). After 12 days, fungal colonies were removed from the agar by peeling the biomass from the dialysis membranes using a sterile razor blade. Mycelia were oven-dried at 100°C until reaching constant weight for at least 2 days. Results were expressed in terms of a tolerance index (TI) as reported in Ceci et al. (2015c). After the dialysis membrane and mycelium were removed, the surface pH of the agar was measured at specific intervals across the diameter of the Petri dish using a conical tip FC 202D pH electrode (Hanna Instruments, Woonsocket, RI, USA) and a pH portable meter, HI 99161 (Hanna Instruments, Woonsocket, RI, USA).

Genetic identification of *P. griseofulvum* FBL 500

P. griseofulvum FBL 500 was isolated and previously identified through conventional taxonomic keys on the basis of macro- and microscopic features. ITS sequence analysis was carried out in order to confirm the previous taxonomical identification findings. *P. griseofulvum* FBL 500 was inoculated by transferring mycelial portions with a flamed glass rod from the actively growing periphery of stock colonies grown in solid Czapek-Dox medium. After 15 days, fungal colonies were removed from the agar by peeling the biomass from the dialysis membranes using a sterile razor blade. Mycelia were freeze-dried, pulverized by using liquid N_2 by using mortal and with the addition of polyvinylpolypyrrolidone to protect DNA. DNA purification and extraction was carried out from single replicates following the standard cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle 1987). Identification of *P. griseofulvum* FBL 500 was achieved after extraction of fungal DNA and ITS sequence analysis using ITS 1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS 4 (5′ -TCCTCCGCTTATTGATATGC-3′) primers for polymerase chain reaction (PCR) analysis (Bellemain et al. 2010). DNA was quantified by using Nanodrop. The PCR amplification was performed in a final volume of 25 µl using: 2.5 µl of 10× buffer, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of dNTPs, 1.5 u. of DNA Taq polymerase (Promega, Milan, IT) and 5 ng DNA. PCR was conducted using 35 cycles of the following reaction conditions: initial denaturation at 95°C for 4 min, 39 cycles of three-step cycling (denaturation at 92°C for 50 s, primer annealing at 55°C for 50 s and extension at 72°C for 50 s) and final extension at 72°C for 10 min. The PCR products were sent to Macrogen Europe (Amsterdam, Netherland) for purification and sequencing. Sequences were edited using the software CHROMAS 2.33 (Technelysium Pty Ltd, Australia). The partial sequence of 18S ribosomal RNA gene, the complete sequence of the internal transcribed spacer 1, the complete sequence of the 5.8S ribosomal RNA gene, the complete sequence of the internal transcribed spacer 2 along and the partial sequence of the 28S ribosomal RNA gene were pairwise compared with those available in the public online databases International Nucleotide Sequence Databases using the BLAST search program (Altschul et al. 1997) and UNITE database (Kõljalg et al. 2005; Abarenkov et al. 2010). The genetic sequence for *P. griseofulvum* FBL 500 was deposited in GenBank with the accession number KY560469.

Experiment A. Metal-amended plates and inoculation

Stock solutions of vanadium pentoxide, V_2O_5 (Riedel-deHaën, Seelze, Germany), ammonium metavanadate, NH_4VO_3 (Merck, Darmstadt, Germany), and lead carbonate, $PbCO_3$ (GPR), were prepared from oven-sterilized aliquots (48 h, 100°C). Growth experiments at different concentrations of ammonium metavanadate (2.5 and 5 mM) were performed. In addition, combinations of insoluble 2.5 mM lead carbonate with insoluble 1.25 mM vanadium(V) oxide or with slightly soluble 2.5 mM ammonium metavanadate were examined to study the toxic effects of both substances on *P. griseofulvum* FBL 500. These concentrations take account of the range of concentrations of these metals found in soils and volcanic

rocks at the same site. The fungus was isolated where vanadium concentrations were over the range 3–6 mM, while lead concentrations were <1 mM (data not shown). Observations of colonies and media were performed using light- and stereo-microscopy to monitor growth, sporulation, pigment production and secondary mineral precipitation.

Experiment B. Batch experiments on HCH biodegradation by *P. griseofulvum* FBL 500

High purity mixture of hexachlorocyclohexane isomers (α:β:γ:δ=1:1:1:1) was acquired from Sigma-Aldrich (Seelze, Germany). Ethyl acetate, acetone and n-hexane were all purchased from ROMIL Ltd (Cambridge, UK) with chemical purity >99.9%. The internal standard γ-HCH-d_6 was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada) and stored at 4°C until use. The tests were carried out at 25°C under shaking conditions at 110 rpm, and with the addition of a mixture of α-HCH, β-HCH, γ-HCH, and δ-HCH isomers (1:1:1:1) to a final concentration of 4 mg/l. Prior to HCH addition, the fungus was grown for 26 days, and the concentration of all isomers and formation of fungal metabolites were monitored at regular intervals over 23 days. Uninoculated sterile flasks with HCH mixture and flasks with fungal culture but without HCH mixture were used as chemical and biological controls. Growth of *P. griseofulvum* FBL 500 and tolerance to the HCH mixture were evaluated by biomass yield. Collected mycelial pellets were oven-dried at 100°C for at least 2 d, until reaching constant weight and fungal tolerance was evaluated using a tolerance index (TI), based on dry weights as described above.

Experiment C. Batch experiments on HCH and vanadium biotransformation by *P. griseofulvum* FBL 500

Tests with at least three replicates were carried out at 25°C in the dark with different combinations of vanadium and HCH isomeric mixture. HCH was directly added in each Petri dish to the final concentration, while Czapek-Dox medium was at a temperature between 40 to 50°C to homogenize HCH in the agar and to avoid HCH volatilization. Growth experiments with insoluble 2.5 mM vanadium(V) pentoxide, and with 4 mg/l and 50 mg/l HCH mixture were performed. In addition, a combination of insoluble 2.5 mM vanadium(V) pentoxide and 4 mg/l HCH mixture was used to study any synergic effects on *P. griseofulvum* FBL 500. The production of fungal metabolites was monitored in biomass and in agar for 20 d at regular intervals. Data of fungal growth (diameter, dry weight) and pH were collected, and tolerance indices were calculated as described above.

Chemical analysis

In experiment B, 5 ml samples were collected from the culture flasks: 1 ml aliquots were spiked with γ-HCH-d_6 as an internal standard and extracted using solid phase extraction (SPE) cartridges packed with Graphitized Carbon Black (GCB — Carbograph, Rome, Italy). HCHs were retained on the solid phase and eluted with 10 ml ethyl acetate through a vacuum manifold (Grayledge Pump & Industrial, LLC, Pelham, NE). In experiment C, the agar and the membranes were collected and analysed to measure HCH

concentration and metabolites. Specimens were posed in glass tubes with 15 mL ethyl acetate and sonicated for 30 min. After centrifugation at 2000 rpm, ethyl acetate solutions were recovered in vials and 10 ml were analyzed.

Residual HCH for each isomer in the culture medium was calculated using the formula reported in Salam and Das (2014) and in Ceci et al. (2015b).

In order to study the uptake of each HCH isomer and fungal transformation, mycelial biomass was collected in all experiments and extracted with Dionex ASE200 Accelerated Solvent Extractor (Dionex, Sunnyvale, USA) after γ-HCH-d_6 addition as an internal standard. Operating conditions are reported in Ceci et al. (2015b). The concentration of isomers and formation of metabolites were analyzed by gas chromatography-mass spectrometry (GC-MS). HCH isomers and the possible metabolites were analyzed using a Hewlett-Packard 6890 gas chromatograph with a 5973A mass selective detector (Agilent Technologies, Palo Alto, California, USA). GC-MS analyses of liquid media were performed to detect any possible intermediate metabolites of dechlorination of HCH (e.g. pentachlorocyclohexene, tetrachlorocyclohexene), or intermediates of HCH reductive dechlorination and hydroxylation as in other studies with bacteria and fungi (Phillips et al. 2005; Guillén-Jiménez et al. 2012).

Analysis of fungal metabolic profile in the presence of HCH mixture and vanadium using Biolog FF microplates

The Phenotype MicroArray™ system (Pinzari et al. 2016) was used to gather information on the whole phenotype of the *P. griseofulvum* FBL 500 strain and on the effects of organic and inorganic toxic compounds on its carbon metabolism. The method we used was based on the inoculation of a fungal spore suspension in FF MicroPlates (Biolog™, Inc., Hayward, California, USA) (Bochner et al. 2001;Pinzari et al. 2016). A combined inoculum of the fungus with: a) 4 mg/l HCH mixture in toluene, or; b) 2.5 mM V_2O_5, or c) with the combination of 4 mg/l HCH mixture and 2.5 mM V_2O_5 were performed in FF MicroPlate™ arrays in triplicate. The inoculation procedure for pure cultures of *P. griseofulvum* FBL 500 in the arrays was based on the protocol used by Tanzer et al. (2003).

Conidia of the fungus were obtained by cultivation of the pure strain on 2% MEA plates in the dark at 25°C for 7 d. Operating conditions are reported in Ceci et al. 2015b. The optical density of Biolog plates was read using a microplate reader (Molecular device, Vmax) at 490 nm (OD_{490}), which was used to measure the intensity of the purple colour resulting from the reduction of the tetrazolium redox dye (*p*-iodonitrotetrazolium), present in the wells of the FF plates, through the action of fungal succinate dehydrogenase as a proxy for respiratory activity. Moreover, optical density at 750 nm (OD_{750}) was used to assess fungal biomass and mycelial growth (Tanzer et al. 2003;Ceci et al. 2015b). Immediately after inoculation, OD_{490} and OD_{750} were measured in order to zero the spectrophotometer specifically for each Biolog plate. Plates were then read at intervals of 24, 48, 72, 96, 168, 192 and 240 h of incubation (Tanzer et al. 2003; Ceci et al. 2015b). In order to evaluate possible redox effects of vanadium pentoxide on the tetrazolium dye, two sets of microplates with V_2O_5 and V_2O_5 with HCH mixture were prepared without the

fungus inoculum. Data obtained from the Phenotype MicroArray™ assays were used to compare the three growth conditions to evaluate the overall differences in metabolism (co-metabolism, inhibition, synergic effects) by studying the utilization of different substrates in the absence or presence of the xenobiotic and PTEs (vanadium and HCH) and their combinations.

Statistical analysis

R elaboration and programming software, version 3.3.2 (The R Foundation for Statistical Computing, Vienna, Austria) and the statistical package XLStat (Addinsoft 2007-Pro, Paris, France) were used to perform statistical analyses (Fahmy and Aubry 2003). They were used to perform one-way ANOVA tests on means for dry weight, diametric growth, surface pH, and HCH concentrations (at least three replicate determinations were used). One-way ANOVA tests on means were performed for the OD_{490} and OD_{750} values for all different treatments at 168 h, when a plateau was reached in the metabolic curves.

The Phenotype MicroArray data were further analysed using the opm R package (Vaas et al. 2013). All the OD values were combined in a dataset which comprised three replicates × 96 substrates × four treatments (control, HCH mixture in toluene, vanadium, combination of vanadium and HCH mixture in toluene) × two metabolic parameters (respiration and mycelial growth), giving rise to 2304 individual phenotypic curves. Comparison of substrate utilization in the different treatments was carried out using the estimated curve parameter of A — maximum height of the metabolic curve — calculated with the opm package and plotted as confidence-interval plots and heatmaps (Vaas et al. 2012). The confidence intervals and the ANOVA were performed on the optical density measurements for 168 h in FF microplates.

Results

Genetic identification of *P. griseofulvum* FBL 500

Sequence analysis using the internal transcribed spacer (ITS) regions (ITS1F and ITS4 primers) confirmed the identification of *P. griseofulvum* FBL 500 through conventional taxonomic keys. A 100% sequence identity over the BLAST alignment was obtained with the name of the reference sequence being *P. griseofulvum* SH207147.07FU and KJ467353 for UNITE and NCBI databases, respectively.

Experiment A. Fungal interactions with metals

The presence of vanadium and lead compounds did not inhibit colony expansion of *P. griseofulvum* FBL 500 under all test conditions. In fact, all the calculated tolerance indices were greater than 1 and colony extension rates in the presence of metal (R_t) showed higher values than the control extension rate (R_c), which was statistically significant in all tests ($P < 0.01$) (Table 2, Online Resource). Multiple comparisons using the Tukey test showed that fungal extension in all tests was significantly higher than the control ($P < 0.01$). Tolerance indices (TI) were used to compare biomass yields of control and test conditions (Table 3, Online Resource). Biomass yields were stimulated (TI > 100%) by the lead and vanadium compounds. The highest TI value occurred with 2.5 mM NH_4VO_3 (TI = 231.49%). Multiple comparisons using the Tukey test revealed that biomass production in all tests was significantly higher than the control ($P < 0.01$). Table 4 (Online Resource) shows the differences (ΔpH) between average surface pH values of uninoculated agar and agar underneath fungal colonies of *P. griseofulvum* FBL 500, growing on Czapek-Dox medium in the different treatment conditions. In uninoculated agar of all experimental conditions, the range of pH medium was 5–5.3. In contrast, for test conditions, the medium pH decreased after growth for 12 days (pH =4–4.3) with the exceptions of those with combinations of vanadium and lead compounds where the pH values were similar to the uninoculated controls. In the latter, the buffering effect of carbonate might have neutralized fungal acidification of the media (Table 4, Online Resource). Sporulation in tests was limited to the central part of the colonies, occasionally green coloured, and the production of yellow-orange pigments and exudates was observed to be higher in treatments than in controls. No secondary mineral precipitation was observed over 12 days. In contrast, the complete dissolution of insoluble crystals of lead carbonate and vanadium pentoxide by *P. griseofulvum* FBL 500 occurred. Notably, few crystals were observed underneath growing fungal colonies in media amended with combinations of ammonium metavanadate and lead carbonate after 3 months incubation of *P. griseofulvum* FBL 500 (Fig. 1). The crystals were red, acicular and tapered, and morphologically resembled lead oxalate. However it was not possible to extract them for further identification because of their limited quantity.

Experiment B. Fungal interactions with HCH mixture of isomers in liquid Czapek-Dox medium

Addition of 4 mg/l isomeric HCH mixture to liquid Czapek-Dox medium had no obvious adverse effects on the growth of *P. griseofulvum* FBL 500. Biomass yield was not strongly reduced (TI > 50%) by the presence of the HCH mixture, and an average TI value of 87.2% was obtained. Results from the time-

dependent studies of HCH concentration in liquid batch tests are shown in Fig. 2. No measurable changes in isomer concentration were detected in the abiotic controls throughout the experiments. The HCH mixture was added 26 d after fungal inoculation, and the isomer concentrations were monitored for 23 d. Up to the third day, the isomer concentration increased and reached a maximum concentration for all the isomers. This phase was followed by a reduction of α-HCH, β-HCH, and γ-HCH in the medium with residual α-HCH = 63.0%, residual β-HCH = 67.1%, and residual γ-HCH = 63.5% (Fig. 2). In contrast, the δ-HCH concentration appeared to be stable and close to the initial concentration of ~1 mg/l. According to ANOVA, there was a significant difference ($P < 0.01$) between the means of the γ-HCH concentrations measured during the second and third day. There was a significant difference ($P < 0.05$) between the means of α-HCH and the β-HCH concentrations measured over the same days. At the end of the experiment, the concentrations of α-HCH, β-HCH, γ-HCH and δ-HCH in the solution were respectively 0.56 ± 0.02 mg/l, 0.76 ± 0.04 mg/l, 0.48 ± 0.02 mg/l and 0.92 ± 0.02 mg/l with a resulting substantial deficit with regard to the initial value of 1 mg/l for each isomer.

Experiment C. Fungal interactions with HCH mixture of isomers, vanadium and combinations of V and HCH in solid Czapek-Dox medium

The presence of 4 mg/l HCH mixture in Czapek-Dox agar medium had no apparent adverse effects on the growth of *P. griseofulvum* FBL 500. The fungus showed a slight reduction of the extension rate in comparison to the control (significant at $P < 0.01$) (Table 2, Online Resource). Biomass yields were not reduced (TI > 70%) by the HCH mixture (Table 3, Online Resource). The TI value was 82.8%, and the difference in biomass yields was statistically significant ($P < 0.01$). Table 4 (Online Resource) shows the differences (ΔpH) between average surface pH values of uninoculated agar and agar underneath fungal colonies of *P. griseofulvum* FBL 500, growing on Czapek-Dox medium in the different treatment conditions. The ΔpH revealed a low medium acidification (Table 4, Online Resource). After growth of *P. griseofulvum* FBL 500, the inoculated medium pH measured in the test condition (6.67 ± 0.19) was slightly higher than the one of the control (6.31 ± 0.19) and statistically significant ($P < 0.01$).

50 mg/l HCH mixture had a toxic effect on growth of *P. griseofulvum* FBL 500 with the extension rate being significantly reduced (Table 2, Online Resource). Biomass yields were strongly reduced (TI < 25%) (Table 3, Online Resource). The TI value was 23.8%, and the difference in biomass yields was statistically significant ($P < 0.01$). After growth of *P. griseofulvum* FBL 500, the average pH value of inoculated medium pH measured in the test (6.73 ± 0.11) was slightly lower than the one of the control (6.31 ± 0.19) and statistically significant ($P < 0.01$).

The addition of 2.5 mM V_2O_5 to Czapek-Dox did not result in adverse effects on the fungal extension rate in comparison to the control, although the differences were not statistically significant (Table 2, Online Resource). Biomass yields were stimulated (TI > 100%) by the presence of vanadium pentoxide (Table 3, Online Resource). The TI value was 123.7%, although the difference in biomass yield was not statistically significant. After growth of *P. griseofulvum* FBL 500, the average pH value of inoculated

medium pH in the test (5.98 ± 0.41) was slightly lower than the one of the control (6.31 ± 0.19) and statistically significant ($P < 0.01$); ΔpH was slightly negative (Table 4, Online Resource).

The combination of V and HCH mixture in Czapek-Dox agar showed a slight reduction of the extension rate in comparison to the control, although differences were not significantly different (Table 2, Online Resource). Biomass yields were not reduced (TI > 70%) by the HCH mixture (Table 3, Online Resource). The TI value was 88.1%, although the difference in biomass yields was not statistically significant. The average pH value in the test (6.24 ± 0.26) was slightly lower than the control (6.31 ± 0.19), although it was not statistically significant. ΔpH was slightly negative (Table 4, Online Resource).

Fungal metabolites from HCH biotransformation

Different metabolic intermediates were observed in experiments B and C (Fig. 3) and different isomers of pentachlorocyclohexene (PCCH) tetrachlorocyclohexene (TCCH), trichlorobenzene (TCB), dichlorobenzene (DCB) and chlorobenzene (CB) were detected. In experiment B, only PCCH was always detected during all the monitoring period in the solid Czapek-Dox medium experiments, while no metabolites were found in liquid Czapek-Dox medium. In experiment C, after 12 d fungal growth, PCCH was found in all tests in the presence of HCH mixture, i.e. 4 or 50 mg/l HCH mixture and the combination of HCH mixture and vanadium. TCB was found only in the combination of HCH and vanadium and in the experiment with 50 mg/l HCH mixture. In the latter, all metabolites (PCCH, TCCH, TCB, DCB, CB) were detected. Moreover, the benzoates, benzaldehyde and benzyl alcohol, were also detected during the experiments.

Analysis of fungal metabolic profile in the presence of HCH mixture and vanadium using Biolog FF microplates

The Phenotype MicroArray^{TM} system (Biolog Inc., Hayward, CA, USA) was used to investigate the whole phenotype and nutrient utilization by *P. griseofulvum* FBL 500 in both control and test conditions. *P. griseofulvum* was able to grow in the presence of 71 substrates out of the 96 available in the FF plates (Pinzari et al. 2016). A heatmap of A values — the maximum height of the growth curve — for all treatments and all substrates at 750 nm is presented in Fig. 4. The clustergram above the heatmap shows clusters of substrates that refer to the different intensities of fungal metabolism according to the A values measured. The clustergram on the left side shows clusters of treatments in which vanadium and combinations of vanadium and HCH are together, as well as the control and HCH mixture, resulting in a different general response of fungal metabolism to the presence of the xenobiotics. The confidence intervals and the ANOVA were performed on the optical density measurements at 750 nm for the estimation of mycelial growth and at 490 nm for the estimation of respiration after incubation for 168 h in FF microplates in the different treatment conditions — control (no xenobiotics, only fungus), 2.5 mM vanadium oxide, 4 mg/l HCH mixture and combination of both xenobiotics. Significant differences were detected according to specific growth substrates. *P. griseofulvum* FBL 500 was able to use the same substrates in the treatments

as in control conditions, but the presence of xenobiotics influenced fungal metabolism, reducing or increasing respiration rates and/or mycelial growth. The negative effect on respiration and fungal growth was significant ($P < 0.05$) in the presence of vanadium or with a combination of vanadium and HCH for some substrates. In particular, there was a statistically significant reduction of fungal metabolism for D-ribose (pentoses), rhamnose (hexoses), D-gluconic acid (sugar acids), *N*-acetyl-D-glucosamine (hexosamines), maltose, maltotriose, D-melibiose, D-raffinose (oligosaccharides), all considered glucosides with the exception of arbutin, D-salicin and sucrose, all nitrogen-containing compounds and all the biochemical group "other" with the exception of L-asparagine, L-phenylalanine, L-pyroglutamic acid, L-threonine, ethanolamine, malic acid, and sebacic acid (Fig. 5). For all other substrates (36 — 50 %) used by *P. griseofulvum* FBL 500, there was no significant difference ($P > 0.05$) in respiration and growth between control and test conditions. It is worth mentioning that for several substrates, HCH and V (alone or in combination) affected fungal metabolism as revealed by calculated confidence intervals and the absorbance curves for respiration and growth. In particular, for L-sorbose, D-mannitol, L-asparagine, i-erythritol and L-threonine, vanadium influenced fungal metabolism, increasing respiration and growth compared to the control (Fig. 5). In contrast, for D-mannitol, D-cellobiose, D-sorbitol and D-glucosamine, the presence of HCH inhibited fungal metabolism (Fig. 5). For ethanolamine, L-phenylalanine and D-salicin, HCH increased respiration and mycelial growth (Fig. 5). For sebacic acid, the presence of HCH and the combination of HCH and V resulted in metabolic stimulation (Fig. 5).

Discussion

This work examines the potential of *P. griseofulvum* FBL 500, which was isolated from polluted sites, to tolerate high concentrations of the potentially toxic metals, vanadium and lead, and hexachlorocyclohexane, and mediate their biotransformation.

Hexachlorocyclohexane (HCH) is a persistent organic pollutant (POP) of global concern with potentially toxic effects on humans and ecosystems. It is a halogenated xenobiotic which has been reported to be carcinogenic and an endocrine disrupter for humans and other organisms (; Ceci et al. 2015b). Three isomers of hexachlorocyclohexane, α-HCH, β-HCH and γ-HCH, were included as persistent organic pollutants in the 2008 Stockholm Convention because of their worldwide spread and environmental persistence (Vijgen et al. 2011).

Vanadium is considered to be the one of the most abundant elements and one of the most important metals in modern technology (Rehder 2008; Ceci et al. 2015c). Vanadium is also essential for certain organisms (e.g. some algae, bacteria, fungi and lichens) as a cofactor of enzymes and a constituent of metabolites (haloperoxidases, nitrogenases and amavadin) (Crans et al. 2004). In recent decades, hydrocarbon fuel combustion, industrial activities and mining have increased the vanadium concentration in the environment, raising concern over its spread and toxicity for humans and ecosystems (Rehder 2008; Ceci et al. 2015c).

P. griseofulvum has been found to successfully tolerate and accumulate potentially toxic metals such as Cu and Cr (Shah et al. 1999; Shi et al. 2011; Abigail et al. 2015), to tolerate and mediate the biotransformation of Cu-based wood preservatives (Bridbžiuviene and Levinskaite 2007) and Ni and V porphyrins (Cordero et al. 2015). Moreover, this fungus was reported to tolerate high concentrations of pyrene and mediate its biotransformation (Ravelet et al. 2000).

In experiment A, vanadium and lead compounds (NH_4VO_3, V_2O_5, $PbCO_3$) were used. Vanadium(V) oxide and ammonium metavanadate are important products of industrial metal recovery (Teng et al. 2006). Vanadium(V) oxide is often found in leachates from mining and milling activities that account for the most significant fluxes of vanadium in the environment and can also originate from fossil fuel combustion as an insoluble by-product (Teng et al. 2006). Lead carbonate (cerussite), a common insoluble lead mineral in soil, has been used in metal tolerance tests to evaluate possible combined effects of the two metals on *P. griseofulvum* FBL 500 and to evaluate possible metal biotransformations mediated by the fungus (Ceci et al. 2015c). *Aspergillus niger* has been tested with the same metal compounds in similar research on vanadium geomycology (Ceci et al. 2015c). In this work, *P. griseofulvum* FBL 500 showed good growth in experiment A (Table 2 and 3, Online Resource). The presence of ammonium added in the test medium could explain the higher growth rates and biomass yields, being used as an additional N source. However, ammonium was not present with the combination of lead carbonate with vanadium(V) oxide where the extension rate and biomass yield were also significantly higher than the control. Moreover, in the presence of vanadium(V) oxide and sucrose in experiment C, the TI was particularly high. Higher tolerance indices than the control may be related to metabolism-dependent or -independent mechanisms of

tolerance/resistance, implemented to cope with the stress due to metal toxicity (Gadd 1993; 2007; Gadd et al. 2012). The presence of toxic metals can strongly influence the physiology and morphology of fungal mycelia and the resulting interactions can include acidolysis, complexolysis, redoxolysis, metal accumulation, production of high local concentrations of extracellular enzymes, and other metabolites (e.g. organic acids, siderophores, polyphenolic compounds and pigments), mycelial growth strategies (e.g. explorative growth) and hyphal aggregation (e.g. phalanx growth) (; Gadd et al. , 2014; Ceci et al. 2015a, c). The excretion of organic acids (e.g., oxalic, citric, gluconic, and lactic acid) can be strongly influenced by growth conditions such as the presence of toxic metal minerals, nutrient availability, the C and N sources, pH and buffering capacity of the medium (Gadd et al. 2012; Ceci et al. 2015a, c). *P. griseofulvum* FBL 500 was able to acidify the medium beneath the colonies either from the initial value of pH 5.5 or from an initial value of pH ~7 (Table 4, Online Resource). In experiment A, the final pH values in all test conditions were all acidic (pH 4–5), with the only exception being the treatments with the combinations of Pb and V compounds. These were close to the uninoculated control and this is probably related to the buffering effect of carbonate (Table 4, Online Resource). Generally, fungi lower the pH of their medium during growth. Mechanisms such as the excretion of protons via the plasma membrane ATPase, the uptake of essential cation nutrients in exchange for protons, the release of organic acids and acidification due to fungal respiration can all cause acidification (Ceci et al. 2015c). On the other hand, in the presence of specific metal compounds such as carbonates or apatites, buffering effects can be evident (Ceci et al. 2015a, c). In this work, the formation of new biominerals, which are assumed to be lead oxalate, is evidence of metal biotransformation. Similar results were observed with *A. niger* in the presence of V and Pb compounds, vanadinite and mimetite (Ceci et al. 2015a, c). The organic acids produced by *P. griseofulvum* FBL 500 can provide ligands for metal-complex formation, electrons for metal redox reactions and metal precipitation as new mycogenic minerals, that can result in mobilization or immobilization of lead and vanadium (Ceci et al. 2015a, c). In experiment C, the medium pH values were close to the control (Table 4, Online Resource). The fungal growth in such conditions did not significantly change the pH of media (Table 4, Online Resource), and a pH range close to neutrality could avoid some possible toxic effects in acidic conditions due to the production of benzoates during HCH biodegradation. The benzoates detected during these experiments were previously observed with *P. griseofulvum* FBL 500 in the presence of β-HCH (Ceci et al. 2015b).

In experiments B and C, *P. griseofulvum* FBL 500 was able to grow and tolerate high concentrations of a mixture of HCH isomers. The tolerance of this fungus was tested in previous work at a combination of 1 mg/l β-HCH (Ceci et al. 2015b). The presence of a 4 mg/l HCH mixture did not inhibit fungal growth, and similar TI values were found for liquid and solid Czapek-Dox media. Similar results were observed in previous work (Ceci et al. 2015b) confirming that the presence of different HCH isomers had no obvious synergic effects on growth. In liquid medium, the biotransformation of the isomers was observed to be different and isomer-specific, with β-HCH and δ-HCH being the most stable and recalcitrant. These findings also agree with some other biotransformation studies (Willett et al. 1998; Phillips et al.

2005). The biodegradation of different mixtures of α-HCH, β-HCH, γ-HCH and δ-HCH by ligninolytic white-rot fungi has been previously reported (Quintero et al. 2007; 2008; Mohapatra and Pandey 2015). To our knowledge, this work is the first evidence of the biodegradation of a HCH mixture by a saprotrophic fungus, isolated from a historically co-contaminated site with metals and HCH. In Quintero et al. (2007), biodegradation values exhibited by *Bjerkandera adusta* (Willd.) P. Karst. of 91.5%, 94.5%, 78.5% and 66.1% were attained after 30 d for γ-, α-, δ- and β-HCH isomers, respectively. The δ- and γ-HCH isomers were degraded to between 15.1 and 70.8% by six different white-rot fungi tested and the highest β-HCH biodegradation (56.6%) occurred with *B. adusta* (Quintero et al. 2008). In our tests with liquid Czapek-Dox medium, it is possible to hypothesize the existence of a lag phase during the first 3 d followed by a reduction of α-HCH, β-HCH and γ-HCH isomers in the media (Fig. 2a–2c). This could be related to slow HCH solubilization in the media and/or enzyme induction and an adaptation period of the fungus to addition of the HCH mixture. These phases were also observed in *P. griseofulvum* FBL 500 in the presence of β-HCH as an adaptation period associated with time and a specific compound threshold for induction of catabolic enzymes (Ceci et al. 2015b. In a slurry batch reactor using *B. adusta*, Quintero et al. (2007) observed a lag phase during the first 5 d followed by a 7-day period in which the concentration of the HCH isomers α, γ, δ, and β decreased to 73.9%, 57.4%, 40.8% and 28%, respectively. Valentin et al. (2007), using the same slurry reactor system, observed a lag period of 6 d, but they related this to stressful culture conditions, e.g. high agitation rates, or a decrease in oxygen transfer. Ceci et al. 2015b observed a lag period of 4 days, followed by a reduction period of β-HCH in the medium (residual β-HCH = 44.8%). Detrimental conditions, such as benzoate formation, could speed up the decay phase, which implies that degradation stops at shorter treatment periods (Guillén-Jiménez et al. 2012; Ceci et al. 2015b). In liquid medium, no expected metabolites that have been reported in the literature for HCH biodegradation were observed, while different metabolites derived from fungal dehalogenation of HCH were observed in experiment C with HCH (Fig. 3). In liquid media, possible intermediates could not be detected probably because of the fast kinetics of biodegradation involved in liquid medium, the low detection limits of the transient products, or their different values of polarity and volatility (Guillén-Jiménez et al. 2012; Ceci et al. 2015b). Isomers of PCCH, TCCH, TCB (1,2,3-TCB, 1,3,5-TCB and 1,2,4-TCB), DCB (1,2-DCB, 1,3-DCB and 1,4 DCB), and chlorobenzene were detected in solid Czapek-Dox medium incubated with *P. griseofulvum* FBL 500 in experiment C (Fig. 3). A combination of vanadium and HCH did not significantly inhibit fungal growth, showing similar TI values as those with the presence of HCH alone, while a 50 mg/l concentration of the HCH mixture negatively reduced the fungal growth (TI < 50%). Despite this, in both cases HCH biodegradation was not influenced negatively, as some metabolites were observed. Intermediates of HCH reductive dechlorination and hydroxylation have been observed in other studies on biotransformation of lindane by bacteria and saprotrophic fungi (Phillips et al. 2005; Salam and Das 2015). PCCH is reported to be the first intermediate of HCH dehalogenation in aerobic degradation pathways (Willett et al. 1998; Middeldorp et al. 2005; Camacho-Pérez et al. 2012). The presence of TCCH was generally observed during anaerobic HCH degradation by bacteria, while a *Pseudomonas* sp. isolated from

soil was able to transform lindane aerobically producing PCCH and TCCH (Phillips et al. 2005). Microbial degradation of lindane has been reported to produce volatile transformation products such as chlorinated benzenes and phenols (Phillips et al. 2005; Salam et al. 2013). Previously, tetrachlorocyclohexane and tetrachlorocyclohexanol were observed in the biodegradation of lindane by white rot-fungi (Phillips et al. 2005), while PCCH and benzoic acid derivatives were observed in aerobic degradation of lindane by *Fusarium verticillioides* AT-100 (Guillén-Jiménez et al. 2012). γ-PCCH and different metabolites were observed in lindane biodegradation by *Candida* VITJzN04 (Salam and Das 2014), while the proposed degradation route for lindane in *Rhodotorula* sp. is very similar to the one that can be hypothesized in this investigation and γ-PCCH, 1,2,4-TCB, 1,2.DCB, CB and other intermediates were reported (Salam et al. 2013).

The Phenotype MicroArray™ microplate system was used to study the whole phenotype of *P. griseofulvum* FBL 500 in response to the different combinations of V and HCH. This fungus was able to use ~74 % of the all available microplate substrates. This confirms a high metabolic versatility of *P. griseofulvum* FBL 500 in different environmental habitats. The different treatments did not change the number of substrates used, but instead the rate of utilization, with significant effects on fungal metabolism. The presence of vanadium alone or in combination with HCH negatively influenced respiration and growth in the presence of different compounds, according to their different chemical categories. The results showed a strong similarity with the treatments with vanadium and with the combination of V and HCH. This can be explained by V inhibition of specific enzymes which are essential for the activation of different metabolic pathways of substrate utilization and also masks the effects of HCH. For instance, enzymatic V inhibition could be due to chemical similarity of vanadate with phosphate, and the resulting competition for phosphorylation of some enzymes, which, in turn, could reduce utilization of specific substrates and metabolism. It has been demonstrated that vanadium enters *Neurospora crassa* as vanadate, which is a potent inhibitor of the plasma membrane ATPase when cells are growing in an alkaline medium and are depleted for phosphate (Ceci et al. 2015c). Functional and phenotypic studies on microbial communities from metal polluted sites using Biolog microplates with high concentrations of Cu and Zn, showed negative effects on community functions due to metal toxicity, but also pollution-induced community tolerance to metals (Klimek and Niklińska 2007). In particular, the fungal community was shown to be less sensitive to metal toxicity than the co-occurring bacterial community (Klimek and Niklińska 2007). Kong et al. 2006 observed that synergic effects of Cu and the antibiotic oxytetracycline on a microbial community negatively influenced functional diversity, resulting in a significantly stronger negative effect for each substrate group on the utilization potential of carboxylic acids and carbohydrates than those of oxytetracycline or Cu alone (Kong et al. 2006). It is worth noting that in our study it has been found that for nearly 50% of substrates used by *P. griseofulvum* FBL 500 there was no significant difference among the metabolic curves for all the treatments. This may indicate that the mechanisms of tolerance/resistance utilized by the fungus were adequate to cope with the stress imposed by the different combinations of xenobiotics and PTEs. Apparently *P. griseofulvum* FBL 500 could transform metal and/or organic

pollutants into less toxic forms since in their presence it showed the same metabolic profile as the control. In fact, for some substrates higher values of A, the maximum height of the metabolic curve, were observed or higher values of OD for HCH, V and combinations of both. These evidences may represent a stimulatory effect of HCH, V or combinations of both in the co-metabolism of specific organic compounds. In the presence of carbon- and nitrogen-containing compounds, specific metabolic pathways, such as the pentose phosphate pathway and glucuronate interconversion, and the presence of relatively non-specific enzymes, such as laccases and cellulose dehydrogenase saprotrophic can be activated, and fungi, could play roles in the biotransformation of HCH alone or with V (Cameron et al. 2000; Mander et al. 2006; Ceci et al. 2015b). V can help in cancer treatment, because it may be actively involved in oxidative radicals'production by Fenton reactions against the tumor cells (Rehder, 2008). These reactions could also enhance the enzymatic catalyst reactions of HCH biotransformation by laccases or other fungal enzymes, involved also in the biodegradation of other xenobiotics. Moreover, V can activate specific enzymes, such as V-dependent haloperoxidases, that can further enhance the degradation of recalcitrant compounds (Ceci et al., 2015c)

The *P. griseofulvum* FBL 500 strain was isolated from an environment extensively polluted by toxic organic and inorganic compounds over several years. Therefore the fungus occurred in an environment where stress was a constant condition and stimulus for survival. The experiments exposed the fungus to toxic compounds singly and in combination with the responses of the fungus being varied. On one hand, the organism showed a generic response which was the same in both the presence of organic compounds and metals. It is likely that fungus initiated a survival or buffering metabolism generically directed to cope with immediate extreme environmental conditions, apparently without distinguishing between metal or organic stressors. It is worth mentioning that the different treatments did not affect the number of carbon sources used by the fungus, but instead modified their rate of utilization suggesting a significant systemic effect on fungal metabolism, and not just on a single pathway. *P. griseofulvum* FBL 500 was able to acidify the medium and the formation of new biominerals was evidence of the occurrence of metal biotransformation. This suggests that the fungus possesses particular mechanisms of tolerance to metal toxicity. When exposed to HCH, however, the biotransformation of the xenobiotic by the fungus did not significantly change the pH of the media, which remained close to neutrality. This could protect the fungus from toxic effects that occur in acidic conditions due to the production of benzoates from HCH biodegradation. In fungi, pH plays important roles in other ecological aspects. For example, in postharvest pathogens, fungal pH modulation of the host environment regulates a multitude of enzymes implied in fungal pathogenicity (Alkan et al. 2012). Moreover, for *Paecilomyces marquandii* (Massee) S. Hughes, the pH of the medium affects the production of reactive oxygen species during biodegradation of the pesticide alachlor. Neutral pH was favourable both for alachlor biodegradation and for oxidative stress reduction (Słaba et al. 2015). The effectiveness of mycelial protection by anti-oxidative enzymes from oxidative stress was observed to be dependent on the environmental pH, exposure time and fungal growth phase (Słaba et al. 2015). Interestingly, the synthesis of laccase and other extracellular peroxidases by some filamentous fungi was higher in the presence of intracellular oxidative stress (Chanda et al. 2015). Besides

570 a reaction to generic stress, *P. griseofulvum* FBL 500 was also capable of activating pathways to use
571 different substrates, tolerating a high concentration of a combination of HCH and V with a supposed co-
572 metabolic mechanism. In fact, *P. griseofulvum* FBL 500 was able to tolerate high concentrations of a
573 mixture of HCH isomers, an ability described so far only in a few fungal species.
574 In this research, we have provided evidence of fungal tolerance to metals and HCH, and its ability
575 in metal biomineralization, metal mobilization and biotransformation of HCH mixtures of isomers, even in
576 the presence of vanadium at high concentrations. This could therefore be relevant to bioremediation
577 treatments (bioaugmentation, biostimulation) of co-contaminated sites through native fungal species
578 selected for extreme environmental conditions.
579

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

Fig. 1 Biomineralization by *P. griseofulvum.* Biomineral precipitation observed underneath growing fungal colonies in Czapek-Dox agar amended with combinations of 2.5 mM ammonium metavanadate and 2.5 mM lead carbonate after 3 months incubation of *P. griseofulvum* at 25°C in the dark. Scale bar = 0.4 mm. Typical image is shown from several examinations.

Fig. 2 Gas chromatographic analysis of HCH mixture in liquid Czapek-Dox medium after growth of *P. griseofulvum*. The HCH mixture was added 26 days after fungal inoculation, and the isomer concentrations were monitored for 23 days. **a** α-HCH concentration. **b** β-HCH concentration. **c** γ-HCH concentration. **d** δ-HCH concentration. The bars are the standard errors of HCH concentrations of three replicates.

Fig. 3 Proposed biodegradation pathway for HCH in *P. griseofulvum*. The initial reaction is the dehalogenation of HCH to pentachlorocyclohexene, the second is the formation of tetrachlorocyclohexene (TCCH) (3,4,5,6-TCCH), the third is the formation of trichlorobenzene (TCB) (1,2,3-TCB, 1,3,5-TCB and 1,2,4-TCB were detected), the fourth is the formation of dichlorobenzene (DCB) (1,2-DCB, 1,3-DCB and 1,4 DCB were detected), and the fifth is the formation of chlorobenzene.

Fig. 4 The parameter "A" (=asymptote) namely the maximum cumulative growth of the fungus on each substrate/in each well, clustered according to the different treatments and visualised as a heat map. The heat map was obtained using the function heat_map of the opm package. The x-axis corresponds to the substrates clustered according to the similarity of their values over all treatments; the y-axis corresponds to the plates clustered according to the similarity of their values over all substrates. The central rectangle is a substrate × plate matrix in which the colours represent the classes of values. Deep violet to blue indicate low optical density values and light brown indicates high values. The four treatments were cont=control, HCH= hexachlorocyclohexane mixture, V= vanadium and HCH+V= combination of vanadium and HCH for measurements at 750 nm.

Fig. 5 Growth curves of *P. griseofulvum* measured at 750 nm (**a**) and 490 nm (**b**). Growth curves for the four treatments (cont=control; HCH= hexachlorocyclohexane mixture; V= vanadium; HCH+V= combination of vanadium and HCH) for some of the most representative carbon sources among the 95 substrates present in FF arrays were elaborated using the opm package for R.